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GENETIC VARIATIONS IN THE MODE OF STOLON GROWTH IN THE HYDROID, *CAMPANULARIA FLEXUOSA* [1]

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A previous investigation into the manner of stolon elongation in *Campanularia flexuosa* (Wyttenbach, 1968) elucidated a cyclic pattern of growth not previously examined in other essentially one-dimensional systems. As originally reported in stolons of *Clytia johnstoni* by Hale (1964), tips were found to elongate by means of an unending series of alternating forward surges and backward partial retractions. A detailed analysis of this pattern in a single genetic stock of *C. flexuosa* revealed that all of the major features of the growth cycle are predictable, in some respects precisely so. It also determined the effects of several environmental factors on this pattern. The present communication extends these observations to a number of additional stocks of this species for the purpose of defining the role which heritable factors may play in modifying the cycle.

In the one stock already described (Wyttenbach, 1968), it was noted that after attaining the crest (the end of a forward thrust), the stolon tip almost immediately retracted slightly (0.5–1.0 μ). Subsequently, a more extensive retraction of variable extent (up to more than 25 μ) occurred. After a brief resting interval, the tip re-extended to approximately the level of the previous crest; then, after another short resting period, it extended forward again to reach a new crest. In this particular stock, the crest-to-crest interval (cycle time) was highly consistent both from cycle to cycle in a given stolon and among different stolons, and averaged 6.15 ± 0.05 minutes. In addition, although the crest-to-crest distance (growth per cycle) routinely varied $\pm 20\%$ from one cycle to the next, nearly all established stolons (those at least 6 days old) grew at an average rate of 19.0–21.5 μ per cycle. Although the extent of retraction following each crest differed considerably from cycle to cycle, it was seen to change consistently from shallow $\leftrightharpoons$ deep on a regular basis, with a period of 5 cycles. It is upon this background of information that the following observations expand.

Materials and Methods

Observations are reported on 19 genetic stocks of *C. flexuosa*. In the present context, a genetic stock is defined as a group of colonies started with cuttings taken from the same wild colony and subcultured as necessary with cuttings from

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one of these laboratory colonies. Therefore, the several stocks observed here were distinguished as genetically different on the premise that wild colonies growing on separate pieces of substrate would be derived from different planulae even when obtained at the same general collecting site.

Colonies were established in the laboratory following the method of Crowell (1957) and are detailed in Wyttenbach (1968). The microscope slides bearing these colonies were suspended in filtered, continuously-flowing sea water adjusted to 20.0 ± 0.5° C. Feeding was carried out by placing the slides twice daily for five minutes each into a dense suspension of newly-hatched *Artemia* nauplii, a method previously found (Crowell, 1957) to provide for optimal growth. To assure that just maximally growing stolon tips were studied, only "established" stolons, those at least 6 days old, were selected for microscopic observation.

Elongation of the stolon tip was followed microscopically by measuring its position with an ocular micrometer at successive 12-second intervals throughout the observation period. By using reflected lighting and black background at a magnification of 125 ×, and by observing activities under conditions permitting the observer to maintain dark visual adaptation, such readings could be made to an estimated accuracy of ±0.3 μ.

During such observation, the colony was immersed in a dish of filtered sea water which was maintained at 20.0 ± 0.05° C by means of a set of immersed lead coils, through which flowed appropriately warmed or cooled water. In order to avoid the effects of short term variations in sea water composition on cycle time as noted earlier (Wyttenbach, 1968, p. 337), all stolons were "read" in an aliquot of the same carboy of filtered sea water set aside for the purpose at the onset of this study.

The stocks have been arbitrarily lettered in sequence, A through S, from the fastest to the slowest growing. Stock F is that previously described (Wyttenbach, 1968). Stocks E, I, M and O are male, the remainder female. In seven of them (A, F, G, M, N, O and S), at least 4 stolons each were observed microscopically, each stolon for 15 consecutive growth cycles (90–110 minutes). In five (C, D, I, J and K), 1–2 stolons each were observed, each for 8–15 cycles. Such close observation of the remaining seven stocks was limited to a single stolon each, followed for 4–5 cycles. Cycle duration was internally so consistent among all stolons timed that measurements on just 4–5 cycles give substantially as accurate a value as would have been obtained on longer continuous observations, provided that the stolon is equilibrated for at least 15 minutes at the temperature of reading before timing is initiated. All stolons were so equilibrated. In addition, the directly measured daily growth rate, as determined on several additional stolons of each of the 19 stocks, agreed closely with the values calculated on the basis of the observed cycle time and growth per cycle. Therefore, the growth per cycle and per day expressed in Figure 1 may also be considered as accurate regardless of the number of stolon growth cycles directly observed. On the other hand, reliable determination of the periodicity of rhythmic variations in the extent of retraction per cycle required continuous observation of a single stolon tip through at least 7–8 cycles, and so this period is expressed in Figure 1 only for those stocks so observed.

Results

Cycle time

Figure 1A shows a range in cycle time from 6.05 to 7.18 minutes among the several stocks, with a nearly continuous distribution of times within this span. Cycle duration within each individual stock was so consistent, varying no more than ±0.2 minute from cycle to cycle in a given stolon and less than ±0.05 minute in average time among stolons, that any two colonies whose average cycle times differ by 0.10 minute or more may be concluded to be of different stock. On this basis, the 19 stocks may be subdivided into 7–8 groups. For instance, stocks G, J, P and R could not be distinguished among themselves, but they differ significantly from the 15 others.

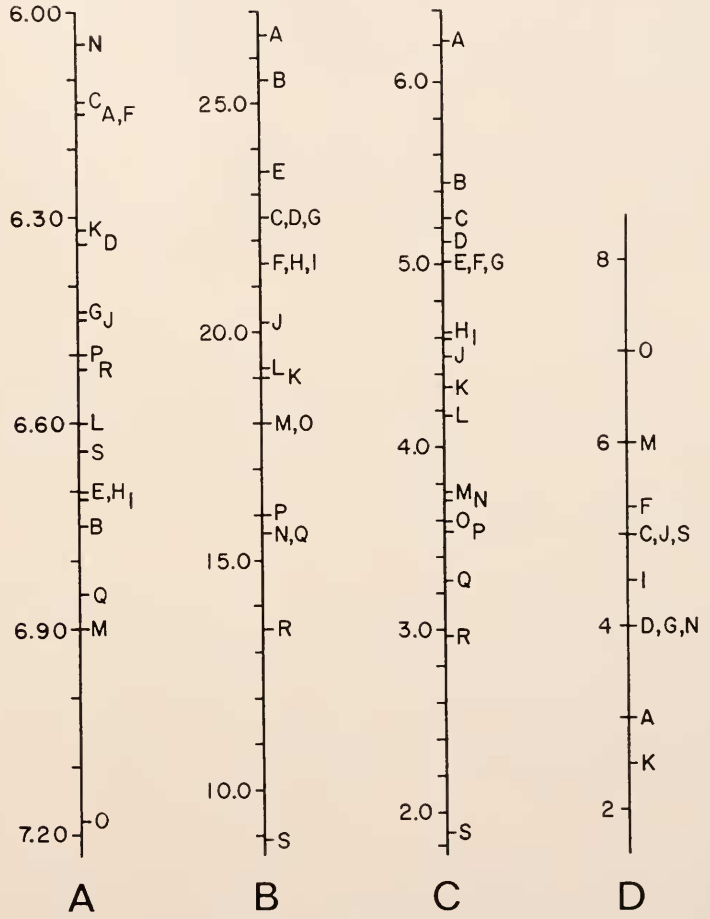

Figure 1. A summary of numerical data relative to stolon growth in 19 stocks (A-S) of *C. flexuosa;* (A) Average cycle duration in minutes, (B) Maximal average growth per cycle in microns, (C) Growth rate in mm per day calculated from values in (A) and (B), (D) Trough period: see explanation in text.

Although cycle duration is a factor in determining the stocks' growth rate, the rankings of the stocks on these two bases show no correlation (compare Fig. 1, A with C). Rapidly growing stock B has a longer cycle than do the two slowest growing stocks, R and S. In addition, the two stocks showing the extremes of cycle time have a virtually identical growth rate.

Growth per cycle

Much greater inter-stock variability is evident in terms of maximal average growth per cycle (Fig. 1B), which shows a 3-fold range, from 8.8 to 26.5 μ. As previously noted in stock F (Wyttenbach, 1968), and confirmed in several additional stocks here, most established stolons have an average per cycle growth at any one time of 90 to 100% of that stock's maximum. Thus by reading 3–4 such stolon tips of a given stock, the highest value obtained should be within at least 5% of the true maximum (*i.e.,* no less than 95% of maximum). In view of this, stocks differing by more than 1.0–1.5 μ per cycle may be distinguished; consequently, with respect to this variable alone, the 19 stocks may again be resolved into 7–8 groups.

The growth rate of the stolon is directly affected by its growth per cycle, and this relationship is clearly evident from a comparison of Figure 1B with 1C. A close parallel in rankings is seen here; the per cycle growth of only stocks E and N are markedly out of line with the ranking sequence of stocks by per diem growth.

Growth per unit time

The greatest variation among stocks occurs in respect to their daily growth rate (Fig. 1C), as calculated from their cycle times and growth per cycle (and corroborated by direct measurements at 24-hour intervals). A more than 3-fold range, from 1.9 to 6.2 mm, is seen between the least and most vigorous stocks. Excepting the extremes, the remaining 17 stocks display a nearly continuous spectrum from 2.95 to 5.45 mm per day.

Based upon the estimated error of no more than -5% in ascertaining the true maximal average per cycle growth of each stock and the possible error of less than $\pm 1\%$ (± 0.05 minute) in determination of its cycle time, the true maximal growth rate should be from -1% to $+6\%$ of that calculated. Thus colonies whose stolons differ by more than 0.35 to 0.40 mm per day in growth rate may be assumed to be of different stock even in the absence of direct measurements of the two component parameters. Here too then, on the basis of growth rate alone, the stocks may be segregated into 6–8 groups.

Retraction "cycle"

Although unrelated to the quantitative aspects of stolonic growth activity, the "trough period" also shows inter-stock variability. Such is illustrated in Figure 2. In Figure 2A, the stolon exhibits shallow retractions in cycles 1, 4 and 7, the others being much deeper, hence a period of 3. The stolon in Figure 2B has a period of 7, with the retractions shallowest in cycle 5, deepest in 1 (and 8). Finally, Figure 2C shows a stolon having a period of 5, with the troughs shallowest

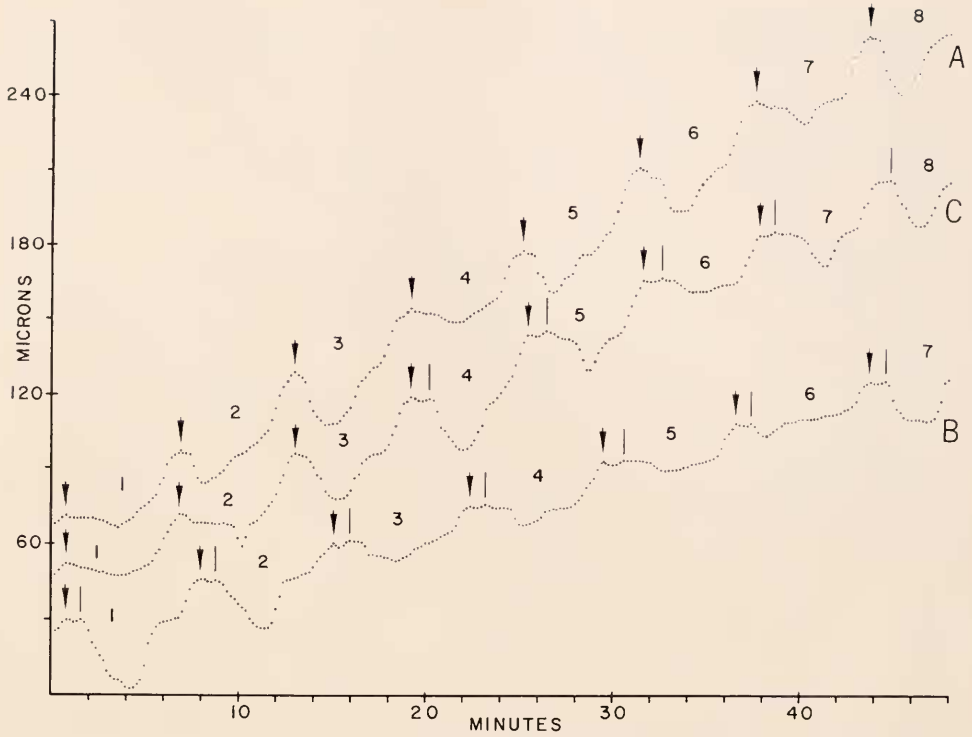

FIGURE 2. The activity of stolon tips of three different stocks. Successive dots are at 0.2 minute intervals except where a crest is reached at an odd tenth of a minute. Arrows denote the crests (primary peaks); pointer lines, the secondary peaks. Numbers 1–8 identify successive cycles, and crests (at left of number), for purposes of text description; (A) Stolon of stock A: cycle time, 6.15 minutes; average growth, 26.5 μ per cycle; trough period, 3 cycles; (B) Stolon of stock O: cycle time, 7.17 minutes; average growth, 16.3 μ per cycle; trough period, 7 cycles; (C) Stolon of stock C: cycle time, 6.14 minutes; average growth, 22.3 μ per cycle; trough period, 5 cycles.

in cycles 1 and 6, deepest in 3, 4 and 8. Figure 1D indicates the trough period of these and nine additional stocks. No consistent numerical relationship exists between this period and either cycle time or growth per cycle. Thus, this trait may serve still further to distinguish differently derived colonies.

Just as noted previously (Wyttenbach, 1968), in every stock, shortly after ingestion of a substantial meal, all retractions are largely suppressed for about 2 hours. They then gradually increase in depth to the maximum (as seen in Fig. 2) by about 8 hours after feeding.

Geometry of the growth cycle

The final feature of stolonic growth which varies among different stocks relates to the pattern of the growth cycle. As originally described (Wyttenbach, 1968) and as illustrated in Figure 2A, following each crest the tip almost immediately (usually within 0.2 minute) retracts by 0.5–1.0 μ, and this slight withdrawal is in

turn followed by the more extensive retraction phase. The stolons of many stocks, however, differ by the appearance of a "secondary peak" following within 0.8 to 1.0 minute after the crest, as illustrated in Figure 2B. This secondary peak represents a resurge of the tip following its slight retraction from the crest, by which it re-extends variously almost to, to, or beyond the previous crest. Such peaks characterize many, most, or all cycles in about two-thirds of the stocks; among the others, they are only seldom seen. No attempt was made to further quantitate their frequency in particular stocks.

One variant on the secondary peak, noted in about one-fifth of the stocks, is diagrammed in Figure 2C. Here such a peak, lacking after crests 1 to 3, appears after crest 4 as a shallow resurge. In subsequent cycles it becomes increasingly dominant to the crest (or primary peak) and the latter then diminishes. By peak 8, the secondary peak has become the definitive crest as the primary peak has disappeared. Consequently, in this stolon in which all other cycles are of 6.0 or 6.2 minutes duration, the cycle of transition measured from primary peak to secondary peak is 7.2 minutes. Such "transition" cycles consitute an exception to the constancy of the cycle time as cited above. These were arbitrarily deleted in the calculation of cycle times shown in Figure 1A. However, since they do not

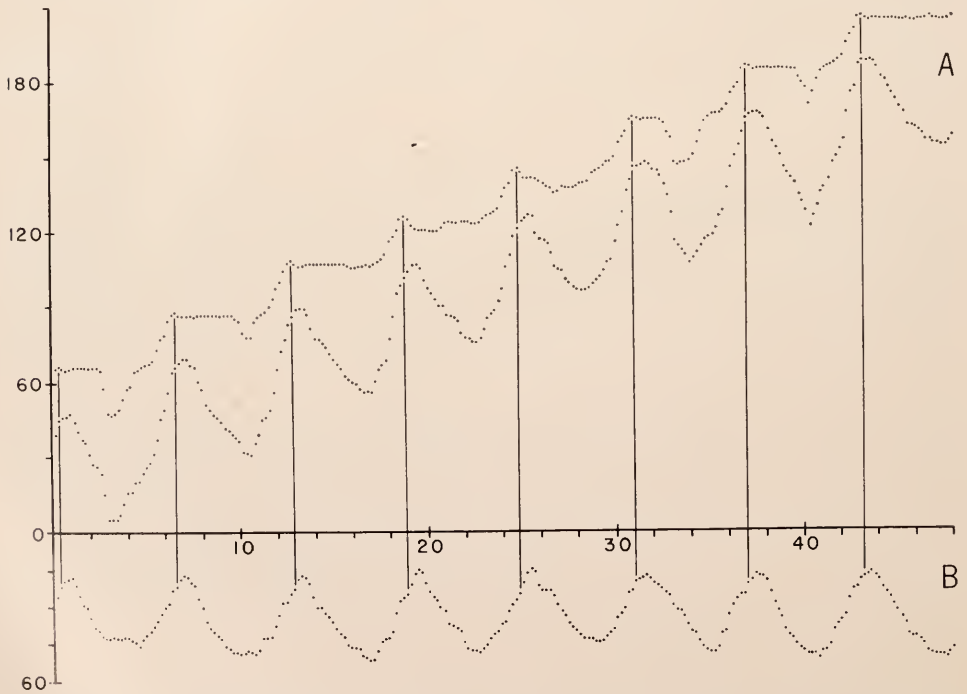

FIGURE 3. (A) Simultaneous observation of both epidermal (upper) and gastrodermal (lower) limit during elongation of a stolon tip of stock F; (B) Epidermal thickness, determined by subtracting the lower from the upper points in (A); Units: x axis, minutes; y axis, microns. Successive dots are at 0.2 minute intervals. Vertical lines align corresponding points at the time of each crest.

appear more frequently than about every 20 cycles in those stocks showing them, their effect on average cycle time would be to lengthen it by only about 0.05 minute.

Secondary peak formation is due to a modification in the relative epidermal-gastrodermal activity at the stolon tip. The epidermis has been shown (Wyttenbach, 1968) to exhibit changes in thickness which are synchronized with the growth cycle. As seen in Figure 3B, it is always thinnest, about 15 μ, at 0.4 to 0.6 minute after each crest. Then, in a stolon lacking secondary peaks, it begins to thicken almost immediately; *i.e.*, by 3–7 μ within the first 0.4 minute, or by 0.8 to 1.0 minute after the crest. Such thickening is correlated with an abrupt retraction of the same magnitude by the gastrodermis (Fig. 3A). When secondary peaks appear, either of two modifications pertains. Usually the gastrodermis retreats only slightly during the 0.4 minute after attaining its farthest advance; alternatively, in a few instances the epidermis thickens more rapidly during this time than shown in Figure 3B. In either event a secondary peak is the result of a short interval when the rate of epidermal thickening exceeds that of gastrodermal retraction; it does not denote a true advance of the coenosarc as a whole. These observations also show that although, as in Figure 3, epidermal thickening parallels gastrodermal retraction, such retraction is not a necessary condition for at least the early phase of thickening. Additional modifications which may occur when a secondary peak becomes the definitive crest have not been sought.

The uniqueness of each stock

Using the combined criteria of cycle time and growth per cycle, only two pairs of stocks are sufficiently similar to suggest identity. Stocks H and I are matched in each measurement, while C and F are within the range of normal variation from each other in each respect. Stocks H and I may not be distinguished either on the basis of trough period or the frequency of secondary peak formation, as H was not observed sufficiently to give clearcut differences from I in measurements of these values. (Yet there is no doubt that H and I differ, as they are, respectively, female and male.) Stocks C and F, however, may be separated in terms of secondary peak formation. Stock C shows secondary peaks which displace the primary peak as the definitive crest (Fig. 2C); on the other hand, stock F, in which over one thousand cycles were observed, forms such peaks only about 25% of the time, and never was one seen to supplant the primary peak as the definitive crest. On the basis of the various stolon features studied, therefore, each of the 19 stocks except H and I is distinct.

Discussion

The collective observations on these several *C. flexuosa* stocks reveal that stolonic growth may differ among them in any of four variables: duration of the growth cycle, average growth per cycle, trough period, and frequency of secondary peak formation. The first two of these together determine the growth rate of the stolon tip, the latter two neither contribute directly to such growth nor show any apparent correlation with growth rate in their expression. In fact, each variable appears to be determined independent of the others.

Cycle time and average growth per cycle influence the growth per unit time disproportionately, as the relative growth rate of the stocks correlates closely with the latter but not at all with the former values. This "dominance" of per cycle growth over cycle time is due to its much greater range of inter-stock variation: the 200% higher per cycle growth of stock A over stock S is many times more than is the 20% longer cycle time of stock O over stock N. Thus stocks A and F, of similar cycle time but differing by about 5 μ in growth per cycle, show a rather large 1.2 mm difference in daily growth. Yet stocks N and Q, which are identical in growth per cycle but are 0.80 minute apart in cycle time, differ in daily growth by only about 0.40 mm.

Rhythmic variations in the depth of retraction from cycle to cycle were described and causally interpreted previously (Wyttenbach, 1968). That evidence, supported by subsequent direct visual observations in *Bougainvillia* (Wyttenbach, in preparation), indicates that the extent of retraction is determined by the nature of the hydroplasmic pressure behind the stolon tip at the time of each retraction phase. This pressure is created by sequential contractions and relaxations of a contractile zone near the stolon tip (Berrill, 1949), and it rhythmically increases and decreases due to alternatingly distal then proximal hydroplasmic flow within the stolon. Such flow follows a generally predictable time table, but *its* cycle time does not equal that for stolon growth. Therefore, the phasing between the two types of cycle is constantly changing. As the retraction phase of the growth cycle occurs at times of successively increasing, then decreasing, hydroplasmic pressure, the retractions become respectively shallower, then deeper. The trough period is therefore determined by the relative cycle times of the two interacting activities, since it represents the frequency with which a given phase relationship is repeated between them.

If the duration of the hydroplasmic flow cycle were the same in all stocks, with variations in the trough period reflecting only inter-stock differences in length of the growth cycle, then stocks with similar growth cycle times would have the same trough period. Such is not the case, as may be seen by comparing Figure 1A with 1D. Consequently, one may conclude that variations in trough period among the stocks reflect differences among them in the average duration of the hydroplasmic flow cycle.

It was previously noted (Wyttenbach, 1968) that despite maintenance of an apparently uniform environment during microscopic observation, there were intervals of days when the cycle time of the stock studied (the present stock F) lengthened by 0.1–0.2 minute. Although the responsible factors are not known, they do *not* provide a basis for the diversity of cycle times among stocks as noted here. Fortuitously, all stocks were observed entirely or in part on days when stock F showed the 6.15 minute cycle. Where some stolons of a few stocks were studied on days of lengthened cycle time in stock F, these also displayed a correspondingly longer interval. Using stock F as a control, it was thus possible to correct these times relative to a figure of 6.15 minutes for F. All values cited in Figure 1A therefore share a common base line.

The four parameters of stolonic acivity described here by no means exhaust the respects in which various *C. flexuosa* stocks may differ. For instance, *Campanularia* pedicels elongate in a fashion similar to stolons (Wyttenbach, Crowell

and Suddith, 1965) and it has been found (Wyttenbach, unpublished) that here too there are inter-stock differences in both cycle time and growth per cycle. Also, the pedicel values may not be predicted from knowing the corresponding stolon values, as they are independently determined. Several additional variable features of colony growth have also been noted, though not measured critically. Thus numerous characteristics of *Campanularia* show inter-stock variation.

As the inter-stock differences observed here are consistent, and the stocks vary only in respect to their initial derivation from separate wild colonies, it is not unlikely that the differences are genetically based. Three of the variables considered, the stolon cycle time, the average growth per cycle, and the cycle time of hydroplasmic flow, are sufficiently easily measured and quantified that they may be amenable to an analysis of their mode of inheritance. The only previous studies of genetic transmission of traits in hydroids are those by Hauenschild (1954, 1956), who concerned himself with the capacity for stolon fusion as expressed in *Hydractinia*. Such fusion occurs between stolons of some pairs of colonies (stocks) but not others, and he has investigated the inheritance of these tissue "compatibility" factors.

Technically, genetic studies in *C. flexuosa* are feasible. In laboratory culture, colonies routinely form numerous gonangia which produce mature gametes continuously for several days or weeks. In addition, young colonies derived from metamorphosed planulae have often been seen when male and female stocks were maintained side-by-side.

Two potential difficulties exist. For one, the stolon cycle duration and growth per cycle, and possibly also the hydroplasmic flow cycle time, vary among the stocks almost continuously between the extremes noted, suggesting that these features may be polygenic in determination and thus more difficult to analyze genetically. Also, since these stocks are not inbred, *i.e.*, are not genetically homozygous, interpretation of the results must be viewed with the realization that diverse genetic backgrounds might affect the expression of genes determining the trait considered. Still, suitably designed crosses should provide insight into the inheritance of traits both related and unrelated to stolonic growth.

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Summary

Comparative observations of stolon elongation in 19 genetic stocks of *C. flexuosa* reveal four respects in which such activity may differ, with each varying independently of the others. Two of these determine the stolonic growth rate: the duration of the growth cycle and the average growth per cycle. The stocks show a nearly continuous spectrum of these values, ranging, respectively, from 6.05 to 7.18 minutes and from maxima of 8.8 to 26.5 μ. The resulting maximal daily growth of the stocks varies from 1.9 to 6.2 mm. This rate is influenced much more by the stolon's growth per cycle than by its cycle time.

The stocks vary also in the frequency of their repeating pattern of retractions from cycle to cycle; this "trough period" ranges from 2.5 to 7. Thus indirectly

it may be concluded that the duration of each cycle of back and forth hydroplasmic flow in the stolon is also an inter-stock variable.

Finally, the stolons of many stocks show a secondary forward surge of the tip following shortly after the crest of the cycle is reached, and the frequency of such "secondary peak" formation is also a stock characteristic. Its anatomical basis is cited.

With respect to these four parameters of stolon growth activity alone, 18 of the 19 stocks are distinct from one another. It is suggested that several of these traits are so constant and easily measured that studies into their mode of inheritance should be feasible.

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